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(54) Title: EXPRESSION OF HETEROLOGOUS PROTEIN

(57) Abstract: The invention relates to the manufacture of recombinant proteins; vectors for use in the recombinant manufacture of protein and recombinant proteins that are adapted for purification.

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Expression of Heterologous Protein

The invention relates to the manufacture of recombinant proteins; vectors for use in the recombinant manufacture of protein and recombinant proteins that are adapted for purification.

The large scale production of recombinant proteins requires a high standard of quality control since many of these proteins are used as pharmaceuticals, for example: growth hormone; leptin; erythropoietin; prolactin, interleukins; interferon's. Moreover, the development of vaccines, particularly subunit vaccines, (vaccines based on a defined antigen, for example gp120 of HIV), requires the production of large amounts of pure protein free from contaminating antigens which may provoke anaphylaxis. The production of recombinant protein in cell expression systems is based either on prokaryotic cell expression or eukaryotic cell expression. The latter is preferred when post-translation modifications to the protein are required. Eukaryotic systems include the use of mammalian cells, e.g. Chinese Hamster Ovary cells; insect cells e.g. *Spodoptera spp*; fungal/yeast (e.g. *Saccharomyces spp, Pichia spp, Aspergillus spp)* and plants.

The production of heterologous recombinant proteins in an expression system relates to a variety of proteins used in both industrial and medical applications.

For example, "therapeutic polypeptides" include tumour suppressor polypeptides (e.g. p53 polypeptide, the APC polypeptide, the DPC-4 polypeptide, the BRCA-1 polypeptide, the BRCA-2 polypeptide, the WT-1 polypeptide, the retinoblastoma polypeptide (Lee, *et al.* (1987) Nature 329:642), the MMAC-1 polypeptide, the adenomatous polyposis coli protein (United States Patent 5,783,666), the deleted in colon carcinoma (DCC) polypeptide, the MMSC-2 polypeptide, the NF-1 polypeptide, nasopharyngeal carcinoma tumour suppressor polypeptide (Cheng, *et al.* 1998. Proc. Nat. Acad. Sci. 95:3042-3047), the MTS1 polypeptide, the CDK4 polypeptide, the NF-1 polypeptide, the NF2 polypeptide, and the VHL polypeptide.

Therapeutic polypeptides also include "antigenic polypeptides" (e.g. tumour rejection antigens the MAGE, BAGE, GAGE and DAGE families of tumour rejection antigens, see Schulz et al Proc Natl Acad Sci USA, 1991, 88, pp991-993). Antigenic polypeptides also includes polypeptide antigens used in the preparation of vaccines which provide protection against infectious agents. For example, viruses such as Human Immunodeficiency Virus (HIV1 & 2); Human T Cell Leukaemia Virus (HTLV 1 & 2); Ebola virus; Human Papilloma Virus (e.g. HPV-2, HPV-5, HPV-8 HPV-16, HPV-18, HPV-31, HPV-33, HPV-52, HPV-54 and HPV-56); papovavirus; rhinovirus; poliovirus; herpesvirus; adenovirus; Epstein Barr virus; influenza virus, hepatitis B and C viruses. Antigens derived from pathogenic bacteria such as *Staphylococcus aureus; Staphylococcus epidermidis; Enterococcus faecalis; Mycobacterium tuberculsis; Streptococcus group B; Streptoccocus pneumoniae; Helicobacter pylori; Neisseria gonorrhea; Streptococcus group A; Borrelia burgdorferi; Coccidiodes immitis; Histoplasma sapsulatum; Neisseria meningitidis ; Shigella flexneri; Escherichia coli; Haemophilus influenzae.* Antigens derived from parasites such as *Trypanosoma spp, Plasmodia spp, Schiztosoma spp*; and pathogenic fungi such as *Candida* spp.

The development of subunit vaccines (e.g. vaccines in which the immunogen is a purified protein) has been the focus of considerable research in recent years and requires the isolation and purification of the candidate protein from a complex mixture of proteins. The emergence of new pathogens and the growth of antibiotic resistance have created a need to develop new vaccines and to identify further candidate molecules useful in the development of subunit vaccines. There are a number of routes via which vaccines may be administered to an animal. The traditional route is by transdermal injection of the vaccine containing an immunogen that invokes an immune response. An alternative route that is not typically undertaken is an orally administered vaccine. The immunogen contacts the immune system via the mammalian muscosal membrane which lines the small intestine through the activity of so called M cells. The M cells overlie regions of the intestine

referred to as Peyers Patches and function to sample antigens contained in ingested material and are therefore essential components in mediating mucosal immunity. Peyers Patches have been identified as major sites for the adherence and entry of enteric pathogens such as *Escherichia, Salmonella, Yersina and Shigella.*

Plant derived vaccines have been developed which utilise plant oil bodies which have been engineered to present an antigen to which an immune response is desired, see WO01/95934. Plants that bear oilseeds such as soybean, rapeseed and sunflower store triglycerides in their seeds which provide an energy store for germination and subsequent seedling development. Further examples of this are described in WO93/20216; WO96/21029 and WO98/21115.

Therapeutic polypeptides can be "cytotoxic polypeptides" (e.g. pseudomonas exotoxin, ricin toxin, diptheria toxin); or polypeptides which are "cytostatic polypeptides" (e.g. p21, the retinoblastoma polypeptide, the E2F-Rb polypeptide, cyclin dependent kinase inhibitors such as P16, p15, p18 and p19, the growth arrest specific homeobox (GAX) polypeptide as described in Branellec, *et al,* see WO97/16459 and WO96/30385.

Also included within the scope of therapeutic polypeptides are therapeutic antibodies. Preferably said antibodies are monoclonal antibodies or at least the active binding fragments thereof. Monoclonal antibodies may be humanised or chimeric antibodies. A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody. A humanised antibody is produced by recombinant methods to combine the complementarity determining regions (CDRs) of an antibody with both the constant (C) regions and the framework regions from the variable (V) regions of a human antibody.

Plants that bear oilseeds such as soybean, rapeseed and sunflower store triglycerides in their seeds which provide an energy store for germination and subsequent seedling

development. In the seeds the oil globules form oil bodies in the cytoplasm that are membrane bound organelles that have protein in the membrane surface. An example of a protein found associated with oil bodies is an oleosin. The oleosins are a family of proteins that have divergent amino and carboxyl termini with more highly conserved lipophilic central domain. These proteins are strongly associated with oil bodies and it is thought that the association is due in part to the lipophilic domain.

We have cloned two plant lipases referred to as OBL1 and OBL 2 from castor bean that have different lipid specificities. The sequence of OBL 1 and OBL 2 has revealed a lipophilic domain that lacks any homology to that found in the oleosin described in WO93/21320 and represents an alternative means to localise polypeptides which comprise this sequence to oil bodies and thereby facilitate their purification.

According to an aspect of the invention there is provided a nucleic acid molecule that encodes a chimeric polypeptide wherein said nucleic acid molecule comprises a first part selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence which encodes an amino acid sequence as represented in Figure 3;

ii) a nucleic acid molecule which hybridises to the nucleic acid molecule in (i) and which specifically targets said polypeptide to an oil body; and

iii) a nucleic acid molecule which differs from the nucleic acid molecules of (i) and (ii) due to the degeneracy in the genetic code; and a second part that encodes an heterologous polypeptide.

An heterologous polypeptide is a polypeptide to which a targeting domain as herein disclosed has been fused which would not naturally include such a targeting domain.

In a preferred embodiment of the invention said targeting domain is encoded by a nucleic acid molecule comprising a nucleic acid sequence which hybridises under

stringent hybridisation conditions to a nucleic acid molecule which encodes an amino acid sequence as represented in Figure 3.

Stringent hybridisation/washing conditions are well known in the art. For example, nucleic acid hybrids that are stable after washing in 0.1x SSC, 0.1% SDS at 60°C. It is well known in the art that optimal hybridisation conditions can be calculated if the sequence of the nucleic acid is known. Typically, hybridisation conditions uses 4 – 6 x SSPE (20x SSPE contains 175.3g NaCl, 88.2g NaH_2PO_4 H_2O and 7.4g EDTA dissolved to 1 litre and the pH adjusted to 7.4); 5-10x Denhardts solution (50x Denhardts solution contains 5g Ficoll (type 400, Pharmacia), 5g polyvinylpyrrolidone and 5g bovine serum albumen; 100µg-1.0mg/ml sonicated salmon/herring DNA; 0.1-1.0% sodium dodecyl sulphate; optionally 40-60% deionised formamide. Hybridisation temperature will vary depending on the GC content of the nucleic acid target sequence but will typically be between 42^0- 65^0 C.

In a preferred embodiment of the invention said nucleic acid molecule comprises a nucleic acid sequence that has at least 30% homology to the nucleic acid sequence which encodes an amino acid sequence as represented in Figure 3. Preferably said homology is at least 40%; 50%; 60%; 70%; 80%; 90%; or at least 99% identity with the nucleic acid sequence that encodes the amino acid sequence as represented in Figure 3.

According to a further aspect of the invention there is provided a chimeric polypeptide encoded by a nucleic acid molecule according to the invention.

Recombinant protein production relates to the synthesis of protein in expression systems (e.g. bacterial expression systems, yeast expression systems, insect expression systems, mammalian expression systems and plant expression systems). Heterologous polypeptides include commercially important polypeptides, for example enzymes used in biocatalysis (e.g. restriction enzymes, enzymes used in industrial processing; e.g. amylases, proteases, nucleases, lipases) and therapeutic

polypeptides as herein disclosed. The large scale production of recombinant proteins requires a high standard of quality control since many of these proteins are used as pharmaceuticals, for example, interleukins, growth hormone, erythropoietin, interferon. Moreover, the development of vaccines, particularly subunit vaccines, (vaccines based on a defined antigen, for example gp120 of HIV), requires the production of large amounts of pure protein free from contaminating antigens which may provoke anaphylaxis.

In a preferred embodiment of the invention said second part encodes an enzyme.

In a further preferred embodiment of the invention said second part encodes a pharmaceutical polypeptide.

In a yet further preferred embodiment of the invention said second part encodes an antigenic polypeptide. Preferably said second part encodes an antigenic polypeptide associated with an infectious disease condition.

In a preferred embodiment of the invention said second part encodes a polypeptide associated with a viral disease condition.

In a preferred embodiment of the invention said viral disease is the result of an infection caused by a virus selected from the families consisting of: Picornaviridae; Caliciviridae; Togaviridae; Flaviridae; Coronaviridae; Rhabdoviridae; Filoviridae; Paramyxoviridae; Orthomyxoviridae; Bunyaviridae; Arenaviridae; Reoviridae; Birnavirdae; Retroviridae; Hepadnaviridae; Parvoviridae; Papoviridae; Adenoviridae; Herpesviridae; and Poxviridae.

In a preferred embodiment of the invention said virus is selected from the families consisting of: Adenoviridae; Coronaviridae; Herpesviridae; Orthomyxoviridae; Picornaviridae; Poxviridae; Reoviridae; and Retroviridae.

In a further preferred embodiment of the invention said second part encodes a polypeptide associated with a bacterial disease condition.

In a preferred embodiment of the invention said bacterial disease is caused by a bacterial pathogen selected from the group consisting of: Actinomyces; Aeromonas; Bacillus; Bacteroides; Bordetella; Brucella; Compylobacter; Capnocyclophaga; Chlamydia; Clostridium; Corynebacterium; Eikenella; Erysipelothrix; Escherichia; Fusobacterium; Hemophilus; Klebseilla; Legionella; Leptospira; Listeria; Mycobacterium; Mycoplasma; Neisseria; Nocardia; Pasteurella; Proteus; Pseudomonas; Rickettsia; Salmonella; Selenomonas; Shigelia; Staphylococcus; Streptococcus; Treponema; Vibro and Yersnia.

In a further preferred embodiment of the invention said second part encodes a polypeptide associated with a fungal disease condition.

In a preferred embodiment of the invention said fungal disease condition is caused by a fungal pathogen selected from the group consisting of: Aspercillus; Blastomyces; Candida; Cryptococcus; Histoplasma; Phycomyces; and Saccharomyces.

In a further preferred embodiment of the invention said second part encodes a polypeptide associated with a parasitic disease condition.

In a preferred embodiment of the invention said parasitic disease condition is caused by a parasitic pathogen selected from the group consisting of: Trypanosoma; Leishmania; Schistosoma; Plasmodium; Entamoeba; Eimeria; and Trichomonas.

In a preferred embodiment of the invention said parasitic pathogen is selected from the group consisting of: Entamoeba; Eimeria; and Trichomonas.

In an alternative preferred embodiment of the invention said second part encodes a polypeptide associated with cancer.

In a preferred embodiment of the invention said polypeptide is a tumour rejection antigen polypeptide.

In an alternative preferred embodiment of the invention said second part encodes a polypeptide that is an adhesive polypeptide, preferably said adhesive polypeptide is a mussel adhesive polypeptide.

Mussel-derived adhesives possess a variety of characteristics that make them of particular interest to material scientists. The adhesives display a high bonding strength, versatility to a variety of bonding surfaces, resistance and durability, non-toxicity, waterproof and cure without high temperatures properties, characteristics which give these adhesives a range of potential uses and which no man-made adhesive on the market currently possesses. These properties have led to predictions of wide-ranging applications in engineering and medicine. For example a requirement for a natural, strong and waterproof adhesive can be found in the marine industry, where it can be used in ship-building. Medical uses, where such an adhesive, with its further advantages of being neither cytotoxic nor antigenic could be used in orthopaedic and dental applications.

In a preferred embodiment of the invention said mussel adhesive polypeptide is encoded by a nucleic acid molecule selected from the group consisting of:

i) a nucleic acid molecule which encodes a polypeptide comprising the amino acid motif

X-K-X-X-Y-P-X-X-Y-K

wherein X is any amino acid residue;

ii) a nucleic acid molecule which encodes a polypeptide wherein at least one of the residues in said motif is modified by hydroxylation; and

iii) a nucleic acid molecule which encodes a polypeptide which has adhesive properties.

In a preferred embodiment of the invention said adhesive polypeptide comprises the amino acid sequence motif: A-K-P-S-Y-P-P-T-Y-K. Preferably said adhesive polypeptide comprises of at least one motif which has the amino acid sequence A-K-P-S-Y-P-P-T-Y-K. Preferably said adhesive polypeptide consists of a plurality of said motifs linked in tandem.

In a preferred embodiment of the invention said adhesive polypeptide comprises at least 10 motifs, preferably greater than 10 motifs. More preferably still said adhesive polypeptide comprises at least 50 motifs, preferably between 50 and 100 motifs, more preferably between 60-85 motifs.

In a further preferred embodiment of the invention said hydroxylation is to an amino acid at position 6 and/or 7 and/or 9 of at least one motif of the amino acid sequence, X-K-X-X-Y-P-X-X-Y-K. More preferably still said hydroxylation is to amino acid position 6 and/or 7 and/or 9 of at least one motif of the amino acid sequence, A-K-P-S-Y-P-P-T-Y-K.

In a further preferred embodiment of the invention said adhesive polypeptide further comprises a second motif wherein said second motif is represented by the amino acid sequence: X P X P X X X X Y K.

Preferably said second motif is selected from the group consisting of at least one of P P K P T P P T Y K; S P P P P V Y K Y K; or S P P P P T P V Y K, or combinations thereof.

In a further preferred embodiment of the invention said nucleic acid molecule encodes a secretion signal, for example an expansin secretion signal.

In a preferred embodiment of the invention said secretion signal comprises the amino acid sequence: MAFSYSPFSSLFLLPFFFVFTFADYGGWQSGHATFYGGGDASGT,

or functional variant thereof.

In a yet further preferred embodiment of the invention said adhesive polypeptide comprises an endoplasmic reticulum targeting sequence, preferably said sequence is represented by the amino acid sequence HDEL, or functional variant thereof.

In a further preferred embodiment of the invention said second part encodes a spider polypeptide, preferably a spider silk polypeptide.

In our co-pending application WO04/016651, we describe a new spider silk protein. In addition to the tensile strength and elasticity of the silk, the additional characteristics of super contraction, insolubility and non-allergenicity make the use of silk in medical and industrial applications an attractive option. Medical applications include; artificial tendons/ligaments, wound-closure systems, such as vascular wound repair devices, haemostatic dressings, patches, glues and sutures. Industrial applications include; bullet-proof vests, light-weight body armour, cables, ropes and parachute cords, even fishing line. Other advantages in using spider silk rather than the synthetic alternatives such as Kevlar™ is that this fibre is derived from petrochemicals, which are processed under extreme, often polluting conditions and add to the waste accumulating in landfills.

In a preferred embodiment of the invention said spider silk polypeptide is encoded by a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

i) a nucleic acid sequence consisting of the sequence as represented in Figure 4a or 4b;

ii) a nucleic acid sequence which encodes a polypeptide domain comprising the amino acid sequence AGRGQGGYGQGAGG and at least two motifs rich in polyalanine wherein said polyalanine motifs comprise at least 6 alanine amino acid residues;

iii) a nucleic acid sequence which hybridises to the sequence presented in (i) and (ii) above and which encodes a silk polypeptide; and

iv) a nucleic acid sequences that are degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) - (iii) above.

In a preferred embodiment of the invention said domain comprises a motif comprising a repetitive tripeptide sequence, GGX (glycine:glycine: X is any amino acid residue), and at least one SS(serine: serine)-polyalanine sequence.

In a yet further preferred embodiment of the invention said second part encodes a polypeptide with glucosyltransferase activity.

Typically, GTases are enzymes that post-translationally transfer glucosyl residues from an activated nucleotide sugar to monomeric and polymeric acceptor molecules such as other sugars, proteins, lipids and other organic substrates. These glucosylated molecules take part in diverse metabolic pathways and processes. The transfer of a glucosyl moiety can alter the acceptor's bioactivity, solubility and transport properties within the cell and throughout the plant. One family of GTases in higher plants is defined by the presence of a C-terminal consensus sequence. The GTases of this family function in the cytosol of plant cells and catalyse the transfer of glucose to small molecular weight substrates, such as phenylpropanoid derivatives, coumarins, flavonoids, other secondary metabolites and molecules known to act as plant hormones. Other GTases transfers glucosyl residues to peptides and polypeptides.

In a preferred embodiment of the invention said glucosyltransferase is encoded by a nucleic acid molecule selected from the group consisting of:

i) a nucleic acid molecule selected from the group consisting of nucleic acid sequences as represented in Table 1;

ii) a nucleic acid molecule which hybridise to the sequences represented in (ii) above; and which have glucosyltransferase activity; and

iii) nucleic acid molecules consisting of nucleic acid sequences which are degenerate as a result of the genetic code to the sequences defined in (i) and (ii) above.

In a preferred embodiment of the invention said nucleic acid molecules hybridise under stringent hybridisation conditions to the nucleic acid molecules as represented in Table 1.

The GTase sequences disclosed in Table 1 are cDNA sequences to which the oil body targeting domain can be fused typically as an in-frame translational fusion.

In a still further preferred embodiment of the invention said nucleic acid encodes a cleavage site to facilitate the purification of said second part from said first part. Preferably said cleavage site is a proteolytic cleavage site.

According to a further aspect of the invention there is provided a vector comprising a nucleic acid molecule that encodes a chimeric polypeptide according to the invention.

Preferably the nucleic acid in the vector is operably linked to an appropriate promoter or other regulatory elements for transcription in a host cell such as a prokaryotic, (e.g. bacterial), or eukaryotic (e.g. fungal, plant, mammalian or insect cell). The vector may be a bi-functional expression vector which functions in multiple hosts. In the example of nucleic acids encoding polypeptides according to the invention this may contain its native promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a nucleotide sequence upstream from the transcriptional initiation site and which contains all the regulatory regions required for transcription. Suitable promoters include constitutive, tissue-specific, inducible, developmental or other promoters for expression in plant cells comprised in plants depending on

design. Such promoters include viral, fungal, bacterial, animal and plant-derived promoters capable of functioning in plant cells.

Constitutive promoters include, for example CaMV 35S promoter (Odell *et al* (1985) Nature 313, 9810-812); rice actin (McElroy *et al* (1990) Plant Cell 2: 163-171); ubiquitin (Christian *et al* . (1989) Plant Mol. Biol. 18 (675-689); pEMU (Last *et al* (1991) Theor Appl. Genet. 81: 581-588); MAS (Velten *et al* (1984) EMBO J. 3. 2723-2730); ALS promoter (U.S. Application Seriel No. 08/409,297), and the like. Other constitutive promoters include those in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680, 5,268,463; and 5,608,142.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induced gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al* (1991) Proc. Natl. Acad. Sci. USA 88: 10421-10425 and McNellie *et al.* (1998) Plant J. 14(2): 247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) Mol. Gen. Genet. 227: 229-237, and US Patent Nos. 5,814,618 and 5,789,156, herein incorporated by reference.

Where enhanced expression in particular tissues is desired, tissue-specific promoters can be utilised. Tissue-specific promoters include those described by Yamamoto *et al.* (1997) Plant J. 12(2): 255-265; Kawamata *et al* (1997) Plant Cell Physiol. 38(7): 792-803; Hansen *et al* (1997) Mol. Gen. Genet. 254(3): 337-343; Russell *et al.* (1997) Transgenic Res. 6(2): 157-168; Rinehart *et al* (1996) Plant Physiol. 112(3): 1331-1341; Van Camp *et al* (1996) Plant Physiol. 112(2): 525-535; Canevascni *et al* (1996) Plant Physiol. 112(2): 513-524; Yamamoto *et al* (1994) Plant Cell Physiol. 35(5): 773-778; Lam (1994) Results Probl. Cell Differ. 20: 181-196; Orozco *et al* (1993) Plant Mol. Biol. 23(6): 1129-1138; Mutsuoka *et al* (1993) Proc. Natl. Acad. Sci. USA 90(20): 9586-9590; and Guevara-Garcia *et al* (1993) Plant J. 4(3): 495-50.

In a preferred embodiment of the invention said tissue specific promoter is a promoter which is active during the accumulation of oil in developing oil seeds, (for example see Broun *et al.* (1998) Plant J. 13(2): 201-210.

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter. In a preferred embodiment the promoter is an inducible promoter or a developmentally regulated promoter.

Particular vectors are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809).

Vectors may also include selectable genetic marker such as those that confer selectable phenotypes such as resistance to herbicides (e.g. kanamycin, hygromycin,

phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

Alternatively, or in addition, said vectors are vectors suitable for mammalian cell transfection or yeast cell transfection. In the latter example multi-copy vectors such as 2μ episomal vectors are preferred. Alternatively yeast CEN vectors and integrating vectors such as YIP vectors are suitable for transformation of yeast species such as *Saccharomyces cerevisiae* and *Pichia* spp.

According to a yet further aspect of the invention there is provided a method to produce a chimeric polypeptide according to the invention comprising the steps of:

i) providing a cell according to the invention and growth conditions conducive to the production of a chimeric polypeptide according to the invention; and optionally

ii) purifying said chimeric polypeptide from said cell or growth media.

According to a further aspect of the invention there is provided a cell transfected or transformed with at least one nucleic acid molecule or vector according to the invention.

In a preferred embodiment of the invention said cell is eukaryotic cell.

In a preferred embodiment of the invention said cell is a plant cell.

According to a further aspect of the invention there is provided a plant comprising a cell according to the invention.

In a preferred embodiment of the invention said plant is selected from: corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), flax (*Linum usitatissimum*),

alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cerale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Tritium aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Iopmoea batatus*), cassava (*Manihot esculenta*), coffee (Cofea spp.), coconut (*Cocos nucifera*), pineapple (*Anana comosus*), citris tree (Citrus spp.) cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa* spp.), avacado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables and ornamentals.

Preferably, plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea), and other root, tuber or seed crops. Important seed crops are oil-seed rape, sugar beet, maize, sunflower, soybean,sorghum, and flax (linseed). Horticultural plants to which the present invention may be applied may include lettuce, endive, and vegetable brassicas including cabbage, broccoli, and cauliflower. The present invention may be applied in tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper. Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc.

In a preferred embodiment of the invention said plant is an oil seed plant.

Oil seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava been, lentils, chickpea, etc.

In a preferred embodiment of the invention said oil seed plant is safflower.

In a further preferred embodiment of the invention said cell is a prokaryotic cell.

According to a further aspect of the invention there is provided a seed comprising a cell according to the invention.

According to a further aspect of the invention there is provided a vaccine preparation comprising a cell, or a fraction of a cell, according to the invention.

In a preferred embodiment of the invention said preparation is an oil body containing fraction. Preferably said fraction includes a chimeric polypeptide according to the invention associated with oil bodies in said fraction.

In a further preferred embodiment of the invention said preparation includes at least one carrier.

The term carrier is construed in the following manner. A carrier is an immunogenic molecule which, when bound to a second molecule augments immune responses to the latter. Some antigens are not intrinsically immunogenic (i.e. not immunogenic in their own right) yet may be capable of generating antibody responses when associated with a foreign protein molecule such as keyhole-limpet haemocyanin or tetanus toxoid. Certain antigens which lack T-cell epitopes, such as polymers with a repeating B-cell epitope are intrinsically immunogenic to a limited extent. These are known as T-independent antigens. Such antigens benefit from association with a carrier such as tetanus toxoid, under which circumstance they elicit much stronger antibody responses.

In a further preferred embodiment of the invention said preparation includes at least one adjuvant.

An adjuvant is a substance or procedure which augments specific immune responses to antigens by modulating the activity of immune cells. Examples of adjuvant include, by example only, Freund's adjuvant, moray deputies, liposome's. Adjuvant are distinct from carriers.

The vaccine preparations may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. In general, the vaccine preparations are prepared by uniformly and intimately bringing the active polypeptide into association with a liquid stabiliser, a finely divided solid stabiliser, or both, and then, if necessary, shaping the product. Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active polypeptide is typically mixed with at least one inert, pharmaceutically acceptable recipient such as sodium citrate or declaim phosphate and/or one or more: a) fillers or extenders such as starches, lactose, sucrose, glucose, manifold and silica acid; b) binders such as carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose and acacia; c) humectants such as glycerol; d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates and sodium carbonate; e) solution retarding agents such as paraffin; f) absorption accelerators such as quaternary ammonium compounds; g) wetting agents such as cetyl alcohol and glycerol monostearate; h) absorbents such as kaolin and bentonite clay and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycol, for example. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and may also be of a composition such that they release the active ingredient(s) only, or preferentially, in a

certain part of the intestinal tract, and/or in delayed fashion. Examples of embedding compositions that can be used include polymeric substances and waxes.

According to a further aspect of the invention there is provided a method to immunise an animal to at least the second part of the chimeric polypeptide according to the invention, comprising administering an effective amount of a chimeric polypeptide or preparation according to the invention sufficient to stimulate an immune response to said second part.

In a preferred method of the invention said animal is human.

In an alternative preferred method of the invention said animal is selected from the group consisting of: mouse; rat; hamster; goat; cow, horse, pig, dog, cat, or sheep.

In a further preferred method of the invention said immune response is the production of antibodies to said chimeric polypeptide.

In an alternative preferred method of the invention said immune response is the production of T-helper cells which recognise the second part of said chimeric polypeptide.

A preferred route of administration is oral. However, intradermal, subcutaneous, intramuscular or intranasal immunisation is within the scope of the invention.

According to a yet further aspect of the invention there is provided an antibody obtainable by the method according to the invention.

In a preferred embodiment of the invention said antibody is a therapeutic antibody.

In a further preferred embodiment of the invention said antibody is a diagnostic antibody. Preferably said diagnostic antibody is provided with a label or tag.

In a preferred embodiment of the invention said antibody is a monoclonal antibody or binding fragment thereof. Preferably said antibody is a humanised or chimeric antibody.

A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody. A humanised antibody is produced by recombinant methods to combine the complementarity determining regions (CDRs) of an antibody with both the constant (C) regions and the framework regions from the variable (V) regions of a human antibody.

Chimeric antibodies are recombinant antibodies in which all of the V-regions of a mouse or rat antibody are combined with human antibody C-regions. Humanised antibodies are recombinant hybrid antibodies which fuse the complimentarity determining regions from a rodent antibody V-region with the framework regions from the human antibody V-regions. The C-regions from the human antibody are also used. The complimentarity determining regions (CDRs) are the regions within the N-terminal domain of both the heavy and light chain of the antibody to where the majority of the variation of the V-region is restricted. These regions form loops at the surface of the antibody molecule. These loops provide the binding surface between the antibody and antigen.

Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human

diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are therefore thought to be less immunogenic than chimeric antibodies.

It is also possible to create single variable regions, so called single chain antibody variable region fragments (scFv's). If a hybridoma exists for a specific monoclonal antibody it is well within the knowledge of the skilled person to isolate scFv's from mRNA extracted from said hybridoma via RT PCR. Alternatively, phage display screening can be undertaken to identify clones expressing scFv's. Alternatively said fragments are "domain antibody fragments". Domain antibodies are the smallest binding part of an antibody (approximately 13kDa). Examples of this technology is disclosed in US6, 248, 516, US6, 291, 158, US6,127, 197 and EP0368684 which are all incorporated by reference in their entirety.

In a further preferred embodiment of the invention said antibodies are opsonic antibodies.

Phagocytosis is mediated by macrophages and polymorphic leukocytes and involves the ingestion and digestion of micro-organisms, damaged or dead cells, cell debris, insoluble particles and activated clotting factors. Opsonins are agents which facilitate the phagocytosis of the above foreign bodies. Opsonic antibodies are therefore antibodies which provide the same function. Examples of opsonins are the Fc portion of an antibody or compliment C3. Antibodies raised by immunisation and in the form of an immune complex with antigen may bring about opsonisation via the fixation of complement on the antigen, or molecules in its immediate microenvironment.

In a further aspect of the invention there is provided a method for preparing a hybridoma cell-line producing monoclonal antibodies according to the invention comprising the steps of:

i) immunising an immunocompetent mammal with a chimeric polypeptide or preparation according to the invention;

ii) fusing lymphocytes of the immunised immunocompetent mammal with myeloma cells to form hybridoma cells;

iii) screening monoclonal antibodies produced by the hybridoma cells of step (ii) for binding activity to polypeptide of the invention;

iv) culturing the hybridoma cells to proliferate and/or to secrete said monoclonal antibody; and

v) recovering the monoclonal antibody from the culture supernatant.

Preferably, said immunocompetent mammal is a rodent, for example a mouse, rat or hamster.

According to a further aspect of the invention there is provided a hybridoma cell-line obtainable by the method according to the invention.

According to a further aspect of the invention there is provided a method to prepare a vaccine preparation according to the invention comprising;

i) forming a cell extract from a plant cell culture or plant according to the invention; and

ii) separating said extract into an oil body rich fraction.

In a preferred embodiment of the invention said extract is incubated with a protease which cleaves said second part from said first part.

Throughout the description and claims of this specification, the word "comprise" and variations of this word, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

An embodiment of the invention will be described by example only and with reference to the following figures;

Figure 1a is the nucleic acid sequence of OLB1; Figure 1b is the amino acid sequence of OLB1;

Figure 2a is the nucleic acid sequence of OLB 2; Figure 2b is the amino acid sequence of OLB2;

Figure 3 is the amino acid sequence of the hydrophobic domain of OLB 1;

Figure 4a and 4b is the nucleic acid sequence of a spider silk protein; and

Table 1 shows the nucleic acid sequences of plant GTase cDNA's.

Materials and Methods

Materials- Castor beans (*Ricinus communis* var. gibsonii) were supplied by Chiltern Seeds (Ulverston, Cumbria, UK). The beans were soaked in running water for 1 d and germinated in the dark on moist vermiculite at 30°C. All reagents were obtained from Sigma-Aldrich Co. (Gillingham, UK), except for glycerol tri[1-^{14}C]oleate that was from Amersham Biosciences UK Ltd. (Little Chalfont, UK) and *Flavobacterium meningsepticum* propyl endopeptidase (PEP) from Seikagaku America (Falmouth, USA).

Preparation of oil body membranes and proteolytic treatment – Oil bodies were isolated from the endosperm of imbibed castor beans and the membranes delipidated using the methods of Hills et al., (2). Peripheral proteins were removed from the oil bodies using urea buffer, according to Millichip et al., (3). The membrane fraction was solublized in SDS-loading buffer, heated at 70°C for 10 min and the polypeptides separated on a 14% SDS-PAGE gel as described by Laemmli (5) and stained using 0.2% (w/v) Coomassie blue R-250 in methanol: acetic acid: water (4:1:5 v/v/v). For proteolytic treatments purified urea washed oil bodies were incubated in 0.1M phosphate buffer (pH 7) containing 0.2 U ml^{-1} of PEP for 1h at 37°C. The oil bodies were recovered by centrifugation.

Tryptic digestion and peptide sequence analysis - In-gel tryptic digestion was performed by washing the gel pieces in Eppendorf tubes three times with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate and drying the gel pieces in a vacuum concentrator for 30 min. Sequencing-grade modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate containing 0.1% (v:v) octyl-ß-D-glucopyranoside (Sigma) to give a final trypsin concentration is 0.02 µg/µL. Gel pieces were rehydrated by adding 10 µL of trypsin solution, and after 30 min enough trypsin solution was added to cover the gel. Digests were incubated overnight at 37°C.

Tryptic digests were applied directly to the MALDI target plate. Dried peptides were redissolved in 0.5 µL of formic acid followed by addition of 9.5 µL of water, and one or more 0.5 µL aliquots were spotted and dried onto the MALDI target plate. A matrix solution containing 10 mg/mL 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% TFA (v:v) was freshly prepared. Serial 2-fold dilutions of matrix solution were spotted onto the MALDI target plate, and the dilution that gave a uniform layer of crystals when viewed in the mass spectrometer was selected for use with samples. A 0.5 µL aliquot of matrix solution was pipetted onto the dried peptide spot. In some cases, the matrix was added directly to the sample droplet on the target plate before drying.

Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (CTS version, Applied Biosystems, Foster City, CA, USA) in reflectron mode with an accelerating voltage of 20 kV. MS spectra were acquired with a total of 1000 laser pulses over a mass range of m/z 800-4000. Final mass spectra were the summation of 20 sub-spectra, each acquired with 50 laser pulses, and internally calibrated using the tryptic peptides at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data.

For CID-MS/MS, a Source 1 accelerating voltage of 8 kV, a collision energy of 1 kV, and a Source 2 accelerating voltage of 15 kV were used. Air was used as the collision gas at the instrument's 'medium' pressure setting with a recharge threshold of $9.9x10^{-7}$ torr, which produced a Source 2 pressure of about $1x10^{-6}$ torr. The precursor mass window was set to +/-10 "Da", and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra.

Mass spectral data obtained in batch mode were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version1.7). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 1.0) to Mascot. Search criteria included: Maximum missed cleavages, 1; Variable modifications, Oxidation (M); Peptide tolerance, 25 ppm; MS/MS tolerance, 0.2 Da. Peptide sequence tags were generated from CID-MS/MS spectra by manual interpretation or using a *de novo* sequencing program supplied by Applied Biosystems.

RNA extraction, cDNA synthesis and PCR - Total RNA from various tissues was isolated using the RNeasy kit from Qiagen. The synthesis of single stranded cDNA was carried out using SuperScript™ II RNase H⁻ reverse transcriptase from Invitrogen. Degenerate primers corresponding to peptide sequences 1 and 2 were designed (5'-ttgatagtirtyagyttyaga and 5'-ctgtccraatgtrtaiarctt) and used to amplify a fragment of the acid lipase (*RcOBL1*) cDNA from imbibed seed endosperm. Gene specific primers (5'-gaccacttggtatgggcatatgatgg and 5'-catgtcattgcagtaaaccaccctga) were then used to obtain the full-length cDNA sequence by 3'- and 5'-RACE using the SMART™ RACE cDNA Amplification kit from BD Biosciences, following the manufacturers protocols. Primers to a castor actin-like gene (*RcACT*) (5'-cgttctctccttgtatgccagtggtc and 5'-gagctgctcttggcagtctcaagttc) were used as a constitutive control for RT-PCR experiments on various tissues.

A partial clone of *RcOBL2* was obtained by performing PCR on cDNA from four-day old endosperm using different combinations of four pairs of degenerate primers designed using alignments of multiple *OBL-like* genes (5'- rtyagyttyagaggiachgarc or 5'-rtyagyttyagaggiachgayc or 5'-rtyagyttyagaggcachgarc or 5'-rtyagyttyagaggcachgayc with 5'-giaccatrtcrttrttrtabac or 5'-giaccatrtcrttrcartabac or 5'-giaciayrtcrttrttrtabac or 5'-giaciayrtcrttrcartabac). Gene specific primers (5'-taggtctgggcaacagaagtgacgctac and 5'-tgcccaaatgtgtatatgttcagcaacc) were then used to obtain the full-length *RcOBL2* cDNA sequence by 3'- and 5'-RACE using the SMART™ RACE cDNA Amplification kit from BD Biosciences, following the manufacturers protocols.

ABBREVIATIONS

The abbreviations used are: TAG, triacylglycerol; CHAPS, 3-cholamidopropyl-dimethylammonio-1-propanesulphonate; PEP, propyl endopeptidase; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; CID, collision-induced dissociation; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; RACE-PCR, rapid amplification if cDNA ends-PCR; PAGE, polyacrylamide gel electrophoresis; MBP, maltose binding protein.

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>UGT71B1 TABLE 1

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Claims

1. A nucleic acid molecule that encodes a chimeric polypeptide wherein said nucleic acid molecule comprises a first part selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence which encodes an amino acid sequence as represented in Figure 3;

ii) a nucleic acid molecule which hybridises to the nucleic acid molecule in (i) and which specifically targets said chimeric polypeptide to an oil body; and

iii) a nucleic acid molecule which differs from the nucleic acid molecules of (i) and (ii) due to the degeneracy in the genetic code; and a second part that encodes an heterologous polypeptide.

2. A nucleic acid molecule according to Claim 1 wherein said second part encodes an enzyme.

3. A nucleic acid according to Claim 1 wherein said second part encodes a pharmaceutical polypeptide.

4. A nucleic acid molecule according to Claim 1 wherein said second part encodes an antigenic polypeptide.

5. A nucleic acid molecule according to Claim 1 wherein said second part encodes a mussel adhesive polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

i) a nucleic acid molecule which encodes a polypeptide comprising the amino acid motif

X-K-X-X-Y-P-X-X-Y-K

wherein X is any amino acid residue;

ii) a nucleic acid molecule which encodes a polypeptide wherein at least one of the residues in said motif is modified by hydroxylation; and

iii) a nucleic acid molecule which encodes a polypeptide which has adhesive properties.

6. A nucleic acid molecule according to Claim 1 wherein said second part encodes a spider silk polypeptide comprising a nucleic acid sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of the nucleic acid sequence as represented in Figure 4a or 4b;

ii) a nucleic acid molecule which encodes a polypeptide domain comprising the amino acid sequence AGRGQGGYGQGAGG and at least two motifs rich in polyalanine wherein said polyalanine motifs comprise at least 6 alanine amino acid residues;

iii) a nucleic acid molecule which hybridises to the sequence presented in (i) and (ii) above and which encodes a silk polypeptide; and

iv) a nucleic acid sequences that are degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) - (iii) above.

7. A nucleic acid molecule according to Claim 1 wherein said second part encodes a polypeptide with glucosyltransferase activity and is encoded by a nucleic acid molecule selected from the group consisting of:

i) a nucleic acid molecule selected from the group consisting of nucleic acid sequences as represented in Table 1;

ii) a nucleic acid molecule which hybridise to the sequences represented in (ii) above; and which have glucosyltransferase activity; and

iii) nucleic acid molecules consisting of nucleic acid sequences which are degenerate as a result of the genetic code to the sequences defined in (i) and (ii) above.

8. A nucleic acid molecule according to any of Claims 1-7 wherein said molecule encodes a cleavage site to facilitate the purification of said second part from said first part.

9. A nucleic acid molecule according to Claim 8 wherein said cleavage site is a proteolytic cleavage site.

10. A chimeric polypeptide encoded by a nucleic acid molecule according to any of Claims 1-9.

11. A vector comprising a nucleic acid molecule that encodes a chimeric polypeptide according to Claim 10.

12. A cell transfected or transformed with a nucleic acid molecule according to any of Claims 1-9 or a vector according to Claim 11.

13. A cell according to Claim 12 wherein said cell is a plant cell.

14. A method to produce a chimeric polypeptide according to Claim 10 comprising the steps of:

i) providing a cell according to Claim 12 or 13 and growth conditions conducive to the production of a chimeric polypeptide according to the invention; and optionally

ii) purifying said chimeric polypeptide from said cell or growth media.

15. A plant comprising a cell according to Claim 13.

16. A seed comprising a cell according to Claim 13.

17. A vessel comprising a cell according to Claim 12 or 13.

16. A vessel comprising a polypeptide according to Claim 10.

17. A vessel according to Claim 16 wherein said vessel is a bioreactor.

18. A vessel according to Claim 17 wherein said bioreactor is a fermentor.

19. A vaccine preparation comprising a cell, or a fraction of a cell, according to Claim 12 or 13 or a plant according to Claim 15.

20. A preparation according to Claim 19 wherein said preparation is an oil body containing fraction.

21. A preparation according to Claim 19 or 20 wherein said preparation includes at least one carrier.

22. A preparation according to Claim 20 or 21 wherein said preparation includes at least one adjuvant.

23. A method to immunise an animal to at least the second part of the chimeric polypeptide according to Claim 10, comprising administering an effective amount of a chimeric polypeptide or preparation according to any of Claims 19-22 sufficient to stimulate an immune response to said second part.

24. A method according to Claim 23 wherein said animal is human.

25. An antibody obtainable by the method according to the invention.

26. An antibody according to Claim 25 wherein said antibody is a monoclonal antibody or binding fragment thereof.

27. A method for preparing a hybridoma cell-line producing monoclonal antibodies according to Claim 26 comprising the steps of:

i) immunising an immunocompetent mammal with a chimeric polypeptide or preparation according to any previous Claim;

ii) fusing lymphocytes of the immunised immunocompetent mammal with myeloma cells to form hybridoma cells;

iii) screening monoclonal antibodies produced by the hybridoma cells of step (ii) for binding activity to polypeptide of the invention;

iv) culturing the hybridoma cells to proliferate and/or to secrete said monoclonal antibody; and

v) recovering the monoclonal antibody from the culture supernatant.

28. A hybridoma cell-line obtainable by the method according to Claim 27.

29. A method to prepare a vaccine preparation according to any of Claims 19-22 comprising;

i) forming a cell extract from a plant cell culture or plant according to the invention; and

iii) separating said extract into an oil body rich fraction.

30. A method according to Claim 29 wherein said extract is incubated with a protease that cleaves said second part from said first part.

Figure 1a

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Figure 1b>RcOBL1

MDDAGKITSTSHLIVSPDEGTFLDLFKHIVLSDLGSGAKFFRASDQRVPATAAYYSRWPVSVFICKILQL
FQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLIRLKIPKRGEADFISTIGYLDSRMDLHGTPMVSHQA
DEVISNADNPSLKEGHNSKIKGALGNRSLMDLCIMASKLAYENTKVVERVVAEHWKMHFVADYGGMNYFQ
DARNTHAFIFCDKPKDANLIVISFRGTGPFSIPNWCTDFDFSLVGLGDAGSVHVGFLEAMGLGHRNSISS
FETSINTKSPGSITELRKESEMAPDHLVWAYDGVYFLAASTLKGLLKDHKNAKFVVTGHSLGGALAILFT
CILEIQQETEVLDRLLNVYTFGQPRIGNYNLGYFMQNRLNFPERRYFRVVYCNDMVPRVPFDDVFFTFEH
FGTCIYYDSRFFGYFTKEEPSRNPFGIENAISAHITAWWELWRSFILNHVYGAEYKETWESRMFRILGLF
LPGVAAHSPVNYVNSVRLGRELAIPLMSLKMMAQGY

Figure 2 2/4

GATCTTCAAAGTAGTTTTGAACTCTCTGCTGAGGGAAAAAAAGAGTGAGGGAAGTGAGAAACCAAAAGCC
ATGGCTGCTTCTGCTACTACTAGCAATAATATTGCTCCAAACTTCTTGGTTGTTGACCCAAAAAAGGGAA
GAAAAAGAGACATATTCAAGTATTTGGTGAGGAAAGATGTGAAGAGTGGAATGAGTTTCTTGGATAGTTC
AGAGGAAGGAGTTAAAGGTGGCGCAGCAGTTGATCATAGGTGGATTTTATTGGTTTCTATCATCATTCGG
AGGGTTCTTGCGCTTATTGATACCCCATTAAAGTACCTTGGATATGTCATTGATTTCTTTCTCAACCTTA
TCTCCCAAAATAGTGGATTCTCTGGCATACTCAACAACTTTCTCCATGGAAACCTGAAGATACCGAGGAG
AGGAACAGAGAATTTTATAAGCACGATTGGGCAATTGGATGGGCGAATAGACCTTTATAGAACTACAATA
TTATCGGAGAAAGTAGATGATTCTGTTGCTACTGATGTTAACAACATTAAAGCAGAACTGGGTAATCGAT
ATCTCATGGATCTTTGTATCATGGCAGCCAAACTTGTCTATGAGAATGAGAAAGTTGCTCAAAATGTTGT
TGATCGTCACTGGAAGATGCATTTTGTGGCTTTCTACAACTGCTGGAATGAGTACCAAAAGCAAAACAAC
ACCCAAGTGTTCATATGTTGTGACAAGCCAAAGGATGCAAATTTGATAGTGGTCAGCTTTAGAGGAACAG
AACCATTTAATGCACAAGATTGGAGTACGGATTTTGATTTCTCGTGGTATGAAATCCCAAAAGTTGGAAA
GATCCATATTGGATTCTTAGAAGCTTTAGGTCTGGGCAACAGAAGTGACGCTACCACTTTCCAAACTCAC
CTTCAGAGGAAACATACAGGTTTCTTCCATCTAAATGGTGAGTCTGAAGGCAATATGACGGAATGGGCAA
AGAAGAGTGCATACTATGCTGTCGCGTTGAAGCTAAAGAGCTTACTGAAAGAACACAGGAATGCTAAATT
TATAGTCACTGGACATAGTTTAGGTGGAGCACTTGCAATATTGTTCCCGTCAATACTGGTTATACAGGAG
GAGACAGAGATGCTAAACAGGTTGCTGAACATATACACATTTGGGCAGCCAAGAATTGGAGATGCACAGC
TTGGAACTTTCATGGAGTCCCACTTGAATTATCCAGTTACTAGATACTTCAGGGTTGTTTACTGCAACGA
TATGGTGCCTAGAGTGCCTTTCGATGACAAGATTTTCGCTTTCAAGCATTTCGGTACATGTCTTTACTAT
GATAGCCGCTACTTTGGCCGATTTATGGATGAGGAGCCGAACAGAAATTATTTTGGACTGAGACACATAA
TTCCAATGCGGGTGAATGCATTATGGGAACTATTCAGAAGTTTTATGATAACCCATGCACATGGACCTGA
CTACCAGGAGAGTTGGTTCTGCACTCTTTCCAGGGTAGCAGGACTGGTGCTTCCTGGTGTTGCTGCTCAT
AGTCCTATAGATTATGTTAATTCAGTTAGGCTTGGAAAGGAGAGAGTAGCTCCAATGACATCCTTGAAAA
GCTTCGCTCGCAAGTCATAAATCTGGGTTGCACTTGTACTCTTCTTCATGGATGAGACACTGAACACAAA
GGAAAATAATAACAGGGTGCAGTTTAAAATGATCATAAGGGAAATAAATCTTATATTTCTTACTCTTACG
GAAATTTGATAATCTGTGACCTTGTGGTTGTGGGTAGTTCCAATTTAATTTCTTTTTCTTTTCAATAAAA
ATCCTGTACTTCGGTGATAATATGAATTATAGTGTGACTTTTTTGGTTGCCCATAGAAAAAAAAAAAAAA
AAAAAAAAAA

Figure 2b>RcOBL2

MAASATTSNNIAPNFLVVDPKKGRKRDIFKYLVRKDVKSGMSFLDSSEEGVKGGAAVDHRWILLVSIIIR
RVLALIDTPLKYLGYVIDFFLNLISQNSGFSGILNNFLHGNLKIPRRGTENFISTIGQLDGRIDLYRTTI
LSEKVDDSVATDVNNIKAELGNRYLMDLCIMAAKLVYENEKVAQNVVDRHWKMHFVAFYNCWNEYQKQNN
TQVFICCDKPKDANLIVVSFRGTEPFNAQDWSTDFDFSWYEIPKVGKIHIGFLEALGLGNRSDATTFQTH
LQRKHTGFFHLNGESEGNMTEWAKKSAYYAVALKLKSLLKEHRNAKFIVTGHSLGGALAILFPSILVIQE
ETEMLNRLLNIYTFGQPRIGDAQLGTFMESHLNYPVTRYFRVVYCNDMVPRVPFDDKIFAFKHFGTCLYY
DSRYFGRFMDEEPNRNYFGLRHIIPMRVNALWELFRSFMITHAHGPDYQESWFCTLSRVAGLVLPGVAAH
SPIDYVNSVRLGKERVAPMTSLKSFARKS

Figure 3 >RcOBL1 core hydrophobic region (50-105)

ATAAYYSRWPVSVFICKILQLFQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLI

Figure 4a

5' -
GCCGCAGCAGCAGCTGGACAGGGCGGTCAAGGTGGATATGGTGGACTAGGTCAAGGAGGATATGGACAAGGTACAGGAAG

TTCTACAGCCGCCGCCGCCGCAGCCGCCGCCGCCGCCGCAGGTGGACAAGGTGGACAAGGTCAAGGAGGATATGGACAAG

GTGCAGGAAGTTCTGCAGCCGCCGCCGCTGCAGCAGCAGCAGCCGCAGCAGCTGGACGAGGTCAAGGAGGATATGGT

CAAGGTTCTGGAGGTAATGCCGCTGCAGCAGCCGCTGCAGCTGCAGCAGCAGCATCTGGACAAGGAGGACAAGGTGG

ACAAGGTCAAGGTGGATATGGACAAGGTGCAGGAATTTCTGCAGCCGCCGCCGCCGCCGCTGCAGCAGCAGCCGGCGCAG

CAGCTGGACGAGGTCAAGGAGGATATGGTCAAGGTGCTGGAGGTAATGCCGCTGCAGCAGCCGCAGCAGCTGCCGCCGCC

GCCGCAGCTGGACAGGGCGGTCAAGGTGGATATGGTGGACAAGGTCTAGGAGGATATGGACAAGGTGCAGGAAGTTCTGC

AGCCGCTGCCGCAGCAGCAGCTGGACGAGGTCAA - 3'

Figure 4b 4/4

5' -
GCGGCCGCGGCGGCAGCGGCGGCAGCGGCGGCAGGGGGACAAGGTGGACAAGGTCAAGGAGGATATGGACAAGGTGCAGG

AAGTTCTGCAGCCGCCGCCGCTGCAGCAGCAGCAGCAGCAGCCGCAGCAGTTGGACGAGGTCAAGGAGGATATGGTCAAG

GTTCTGGAGGTAATGCCGCTGCAGGAGCCGCTGCAGTTGCAGCAGCAGCAGCATCTGGACAAGGAGGACAAGGTGGACAA

GGTCAAGGTGGATATGGACAAGGTGCAGGAAGTTCTGCAGCCGCCGCCGCCGCCGCTGCAGCAGCAGCCGGCGCAGCAGC

TGGACGAGGTCAAGGAGGATATGGTCAAGGTGCTGGAGGTAATGCCGCTGCAGCAGCCGCAGCAGCTGCCGCCGCCGCCG

CAGCTGGACAGGGCGGTCAAGGTGGATATGGTGGACAAGGTCTAGGAGGATATGGACAAGGTGCAGGAAGTTCTGCAGCC

GCTGCCGCCGCAGCAGCAGCAGCCGCCGCAGCAGCTGGACGAGGTCAAGGAGGATATGGTCAAGGTGCTGGAGGTAATGC

CGCTGCAGCAGCCGCCGCAGCAGGTGGACAAGGTGGACAAGGTCAAGGAGGATATGGACAAGGTGCAGGAAGTTCTGCAG

CCGCTGCCGCAGCAGCAGCTGGA - 3'